

ART. XIII—*The Bacteriological Examination of Drinking Water in Victoria*

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Introduction.

This paper is intended to put forward a standard routine method for the examination of drinking water in Victoria. Most Victorian drinking waters are derived from catchment areas in the watersheds, many of which have been rendered, or are naturally free from human pollution. For this reason, no special treatment (filtration or chlorination) is used as a rule, and in consequence the bacterial count may be relatively high. In such areas, the native fauna is abundant, faecal contamination, as indicated by the presence of organisms of the coli-aerogenes group is also much greater than would be allowed in most countries, but organisms derived from this source are not significant, as they are not of human origin. If the normal bacterial content of a particular water is known from many previous tests, gross changes in the bacterial flora will be easily detected, but results must always be considered in conjunction with the conditions prevailing in the watersheds at the time of sampling.

Collection of Samples.

Water samples should be collected in dry sterile bottles of about 200 c.c. capacity and fitted with rubber stoppers. These should be filled by quickly immersing them about 6 inches below the surface of the stream or reservoir with the mouth toward the current, if any; in still water they should be moved forward so that there will be no risk of contamination from the hand or dipstick.

It is the usual practice to return samples to the laboratory as soon as possible after collection, and, unless they can be examined within a few hours of collection, they must be packed in ice. The following information should be supplied with the sample:—

1. The purpose for which the water is required.
2. The source of the sample. e.g., river, pool, reservoir.
3. The state of the watershed from which it is derived, together with any information regarding purification treatment.
4. Weather conditions at, and prior to the time of sampling; particulars of recent floods or droughts in the area.
5. Date and time of sampling.

Laboratory Examination.

THE PLATE COUNT.

Though the publications of the British Ministry of Health (1936) and the American Public Health Association (1933), do not include the plate count in their standards of purity, both recommend its use when the water is examined regularly, in which case a greatly increased count requires further investigation. It is also of use in studying the efficacy of filtration plants wherein inefficiency may be shown by a high plate count though the coli-aerogenes count be low. In Victorian waters, examined at this laboratory the 20°C. count is usually slightly higher than, but rarely more than four times as high as, the 37°C. count.

The counts are made by placing 0.5 c.c. of the water sample in each of four sterile petri dishes, and adding thereto 10 c.c. of nutrient agar which has previously been melted, and kept at 45°C. for ten minutes prior to pouring. The plate is then rocked gently to and fro and from side to side five or six times in each direction; rotation causes the colonies to be massed near the circumference of the plate, and therefore difficult to count. Two of the plates are incubated at 37°C. and two at 20°C. for two days. They are then counted, with reflected light, using a lens of approximately 2½ diameters magnification. It was the practice to incubate the 20°C. plates for three days, but frequently the count was spoiled by spreading growths, so a 48-hour period has been adopted. Wilson et al. (1936) have pointed out that the mathematical error in the plate count is of the order of 50 per cent. when two plates are used. When we consider also that personal error plays a large part in the count, and that in any case, its actual value is limited, a close approximation is all that is desired. The publication of the American Public Health Association recommends that, "in order to avoid fictitious accuracy and yet to express the numerical results by a method consistent with the precision of the work, the number of colonies of bacteria per ml. shall be recorded as follows:—

Number of bacteria per ml.

1 to 50 shall be recorded as found,

51 to 100 shall be recorded to the nearest 5

101 to 250 shall be recorded to the nearest 25

251 to 500 shall be recorded to the nearest 50

501 to 1,000 shall be recorded to the nearest 100 "

and so on. This recommendation was adopted in routine practice. Both the American Public Health Association and the British Ministry of Health publications recommended also that plates containing between 30 and 300 colonies should be selected for counting unless the plates from undiluted water contain less than 30. Natural Victorian waters usually fall within these limits, excepting after flood rains, so that dilution is unnecessary, and a count of over 300 requires some explanation.

THE COLI-AEROGENES COUNT.

This is regarded as the best available method of detecting pollution in water samples, though it is recognized as being quantitatively inaccurate. The test is divided into two parts, the presumptive test, and the confirmatory test, in which those tubes giving a positive presumptive test are examined for the presence of the coli-aerogenes group.

The Presumptive Test.

A number of media have been suggested for the presumptive test using lactose fermentation as their criterion. It has been claimed in America and England that certain media, e.g., Dominick and Lauter's medium, brilliant green bile, and MacConkey broth—are highly selective for the coli-aerogenes group. Lactose broth, which had been used in this laboratory up to the time of the present investigation, is well known to give false reactions, i.e. lactose fermentation in the absence of the coli-aerogenes group, and so a confirmatory test is necessary to establish the presence of these organisms. In the hope that a medium might be obtained which would eliminate the laborious and time consuming confirmatory test, four media, MacConkey broth, the crystal violet medium of Salle, a synthetic medium, and Dominick and Lauter medium, were tried in comparison with lactose broth. Each tube showing gas within 48 hours was subjected to a complicated confirmatory test.

The results of these experiments are contained in another paper (Atkinson and Wood 1938a) and show that lactose broth is more sensitive to the coli-aerogenes group than any of the other media tried. It gives a larger total number of confirmed positives, and a higher coli-aerogenes count on the majority of samples tested. No medium was found which materially reduced the number of false positives—a result apparently due to the nature of the bacterial flora in Victorian water, as shown by Atkinson and Wood (1938a). The conclusion was therefore reached that lactose broth is the most suitable medium for the presumptive test.

The Confirmatory Test.

It has been decided that no confirmatory test is necessary for tubes which give acid and gas within 24 hours because, of 251 tubes examined, none failed to confirm. These tubes are therefore called "presumptive positives" according to the American definition, with the modification that acid as well as gas production is required. This amended definition is that a presumptive positive is a tube which gives acid and more than 10 per cent. gas within 24 hours. The American Public Health Association defines a doubtful test as a tube which gives gas in 48 hours but not in 24 hours, and we have found that, as they suggest, all these tubes require confirmation, as only 524 tubes confirmed out of

1,056 tubes tested. The technique of the confirmatory test consists of plating a loopful of the tube to be tested on to a suitable medium which will inhibit non-lactose fermenters and thus facilitate the isolation of members of the coli-aerogenes group. Colonies are then picked off from this plate into lactose broth, which should give acid and gas within 48 hours at 37°C. if the tube under test is a true positive. To decide upon the most suitable plating medium, MacConkey agar, Endo, eosin methylene blue, and violet red bile salt agar were tested in parallel and E.M.B. agar was found to be by far the most selective medium. It was therefore adopted for routine use.

The Technique of the Coli-aerogenes Test.

In Victorian waters, organisms of the coli-aerogenes group frequently occur in 1 c.c. and at other times in 0.1 c.c. quantities of the sample, so it is necessary for these quantities to be examined in every test. For the presumptive test, five tubes of double strength lactose broth are inoculated with 10 c.c. of the sample, and five tubes of single strength lactose broth with 1 c.c. and five with 0.1 c.c. These are incubated for 24 hours at 37°C. and all tubes giving acid and more than 10 per cent. gas are recorded as presumptive positives and discarded. The remaining tubes are incubated for a further 24 hours and all tubes showing gas irrespective of acid are recorded as doubtful tests and are subjected to a confirmatory test.

We have found that, owing to the presence of organisms capable of reducing the indicator, non-appearance of acid does not denote the absence of coliform organisms.

The confirmatory test is carried out by sowing a loopful of the doubtful test on to E.M.B. agar as soon as possible after the commencement of gas formation. The plates are then incubated at 37°C. for from 24 to 48 hours and typical colonies—those having black centres and purple translucent margins—are picked off into lactose broth. If no such colonies are present, all types of colony are sown into lactose broth. The lactose broth tubes are incubated for 48 hours, and those showing acid and at least 10 per cent. gas are recorded as confirmed or completed tests according to the American definition.

The Expression of Results of the Coli-aerogenes Test.

A considerable literature exists on the statistical accuracy of the dilution method of estimating bacterial populations. Greenwood and Yule (1917) brought forward a formula for expressing such results as the most probable number of organisms present in a given quantity of water, and McCrady (1918) has published a series of tables from which this can be read off. More recently, Halvorsen and Ziegler (1933-5) have gone thoroughly into the question of accuracy and have shown that the use of five tubes

for each quantity of sample gives a result with a deviation of + 260 per cent. and - 70 per cent. while this deviation decreases rapidly till the number of tubes used is 60 when it becomes almost constant at ± 40 per cent. It is impracticable in this laboratory to use more than five tubes of each quantity, so that the error of sampling is very great. The writers have carried out actual experiments on this sampling error and find that it falls within the limits set by Halvorsen and Ziegler from mathematical considerations. To state an actual case—a sample in which the most probable number as determined by using 40 tubes of each dilution was actually 250 organisms per 100 c.c. gave results ranging from 80 to 650 organisms per 100 c.c. when tested in batches of five tubes per dilution. Similarly in another case where the mean of 30 tubes of each dilution gave 17 organisms per 100 c.c. the extremes of five tube tests were 4 and 45 per 100 c.c. Thus a result of 4 followed by one of 45 organisms per 100 c.c. does not necessarily mean any change in the bacterial flora of the water. It seems preferable therefore to adopt a method of expression which will have wide though admittedly arbitrary divisions and in which, moreover, these divisions may be used as an indication of the quality of the water analysed in Victoria. Such divisions are given by the following scheme, in which the number only of positive tubes is taken into account, and not the quantity of water which they contain:—

1. 0 tubes positive out of 15 inoculated ..	B. coli not found in 50 c.c.
2. 1 or 2 tubes positive out of 15 inoculated	B. coli present in 50 c.c.
3. 3-7 tubes positive out of 15 inoculated	B. coli present in 10 c.c.
4. 8-12 tubes positive out of 15 inoculated	B. coli present in 1 c.c.
5. 13-15 tubes positive out of 15 inoculated	B. coli present in 0.1 c.c.

The first two divisions of this table lie close to the standards of purity required in Britain and America; the third is considered permissible in Victoria in waters from sources free from human habitation, the fourth requires some explanation such as heavy rain, the last should be regarded with suspicion.

False Positive Reactions.

These have been shown (Atkinson and Wood 1938*b*) to be due to masked positives, anaerobes in symbiosis with gram negative bacilli, and synergic reactions involving a pair of organisms, which may be either a Gram negative bacillus plus a streptococcus, or two Gram negative bacilli. It is worthy of note that in Victorian waters there is a tendency for the smaller quantities, 1 c.c. and 0.1 c.c. to yield organisms of the coli-aerogenes group which the 10 c.c. tubes do not. This seems too frequent to be due to chance, and is possibly due to a masking effect, a supposition very difficult to prove; but in such samples it must be borne in mind that the coli-aerogenes count may be higher than that given by the completed test.

Differentiation between Members of the Coli-aerogenes Group.

The advisability of differentiating so-called faecal from non-faecal *B. coli* has not been decided. Work here (Atkinson, 1934) points to the conclusion that differentiation is of little value and Bardsley (1934) concurs with this view. Until further evidence in favour of differentiation is brought forward, it is not thought advisable further to complicate the examination by adopting it in Victoria.

Summary of Procedure for the Routine Laboratory Test.

1ST DAY.—Inoculate each of four 4-inch petri dishes with 0.5 c.c. of water sample, add agar at 45°C., mix thoroughly and incubate two plates at 37°C. and two at 20°C. for 48 hours. Inoculate five tubes of double-strength lactose broth with 10 c.c., five tubes of single-strength lactose broth with 1 c.c. and five with 0.1 c.c. of sample, and incubate for 24 hours.

2ND DAY.—Read the lactose broth tubes and record those giving acid and more than 10 per cent. gas as presumptive positives. Discard these. Re-incubate remaining tubes for a further 24 hours.

3RD DAY.—Count all the plates. Record all tubes giving gas in lactose broth as doubtful positives, and stroke a loopful of each on to E.M.B. agar in 3-inch plates and incubate the plates for 24-48 hours. Discard all negative lactose broth tubes.

4TH DAY.—Select colonies from any plates showing typical positive colonies (black centres and purple translucent margins) and transfer to lactose broth and incubate at 37°C. for 48 hours.

5TH DAY.—Select colonies from remainder of plates, taking all types of colony when no typical lactose fermenters are present, and transfer to lactose broth and incubate for 48 hours.

6TH AND 7TH DAYS.—Record all lactose tubes giving acid and more than 10 per cent. gas in 48 hours as completed tests. The total number of positive tests consists of the presumptive positives + the positive completed tests.

Summary.

Certain aspects of the bacteriology of Victorian drinking waters are discussed and a method of bacteriological analysis is described, which it is suggested might be adopted as a standard, and which is essentially a modification of the method suggested in the publication of the American Public Health Association.

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